Volume 272, number 1,2, 121-124 FEBS 08966 October 1990

Luminol enhanced chemiluminescence of the perfused rat heart during ischemia and reperfusion

C. Kumar, M. Okuda, I. Ikai and B. Chance

Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6089, USA and The University City Science Center, Philadelphia, PA 19104, USA

Received 9 August1990

We show that the production of Luminol reactive oxygen radicals in the perfused rat heart under ischemia and reperfusion can be monitored continuously by measuring the chemiluminescence of Luminol-perfused hearts. Luminol did not affect the monitored physiological parameters of the hearts. Chemiluminescence increased during ischemia and reperfusion. Superoxide dismutase treatment of the heart before ischemia, but not catalase, abolished these increases.

Ischemia; Reperfusion; Heart; Chemiluminescence; Luminol

1. INTRODUCTION

Reactive oxygen radical formation during ischemia-reperfusion is currently considered to be an important cause of tissue damage in the heart [1-3]. Most of the evidence for this is indirect and based on the ability of radical scavengers to decrease the adverse consequences of standardized ischemia-reperfusion protocols [4-6]. Quantitative and continuous measurement of radical production in tissue would enable a rigorous study of the time course of radical production, effect of pharmacological agents on radical production, analysis of any direct correlations between the extent of radical production and tissue damage and the development of rational intervention strategies.

Oxygen radicals and their paramagnetic reaction products with tissue constituents or spin trapping agents can be determined through electron paramagnetic resonance (EPR) of frozen tissue samples [7-10] or using spin trapping agents to form stable derivatives of the radicals followed by EPR examination [11-14]. Taken together, these studies indicate that myocardial radical production increases during prolonged ischemia and that there is a large burst of radical production immediately on the onset of reperfusion.

Another method for detection of reactive oxygen species in tissue is the measurement of the low level chemiluminescence that is emitted by the tissue as a consequence of the reactions of the oxygen radicals. Some of this chemiluminescence may be due to hemoglobin or myoglobin assisted breakdown of reactive oxygen species possibly with concomitant lipid peroxidation [15]. We and others have previously studied the low level chemiluminescence of perfused liver and other similar systems [16,17]. While we have detected chemiluminescence changes during ischemia-reperfusion studies of the heart and liver, the low intensity of the spontaneous chemiluminescence makes quantitative studies difficult.

Oxygen radical production by neutrophils has been studied by monitoring chemiluminescence in the presence of chemicals such as Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) that react with oxygen radicals and produce chemiluminescence with a higher quantum yield [18,19]. The use of such chemical leads to large increases in sensitivity and is well established in cellular immunology. For example, the elegant studies of inhibitor and effector sensitivity of Luminol-enhanced chemiluminescence of monocytes and granulocytes by Dahlgren and coworkers show the involvement of myeloperoxidase reactions in the chemiluminescent process as well as the presence of separate intracellular and extracellular processes [20-22]. We describe here the first application of the Luminol-enhanced chemiluminescence technique to the isolated perfused rat heart during ischemia-reperfusion to study oxygen radical production in this system.

Correspondence address: C. Kumar, Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6089, USA

Abbreviations: LEC, Luminol enhanced chemiluminescence; EPR, electron paramagnetic resonance; LVDP, left ventricular diastolic pressure; LVSP, left ventricular systolic pressure; CF, coronary flow; KH buffer, Krebs-Henseleit buffer; KHL buffer; Krebs-Henseleit buffer containing Luminol; SOD, superoxide dismutase; CAT, catalase

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2. MATERIALS AND METHODS

2.1. *Animal preparation*

Sprague-Dawley rats (280–320 g body weight) were injected with sodium heparin (1000 U/kg i.p.) and anesthetized 30 min later with sodium pentobarbital (50 mg/kg i.p.). The heart was rapidly excised and placed in ice-cold perfusion medium. The heart was then cannulated through the aorta and retrograde perfused as per the Lagendorff procedure at a constant perfusion pressure of 100 cm H_2O. The perfusion buffer used was a modified Krebs-Henseleit bicarbonate buffer solution (KH buffer), pH 7.4, consisting of the following (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.25, $CaCl_2$ 1.25, $MgSO_4$ 1.25, $NaHCO_3$ 24.0 and glucose 11.1. The temperature of the perfusion buffer was 37°C. The perfusion buffer was gassed continuously with a mixture of 95% O_2, 5% CO_2 and was filtered through a 0.45 μ Millipore filter. A teflon catheter was inserted into the left ventricle through the left atrium to monitor the left ventricular systolic (LVSP) and diastolic (LVDP) pressure. Coronary flow (CF) was measured by collection of the effluent perfusate at timed intervals. Chemicals and enzymes used were obtained from Sigma. The specific activity of SOD (from bovine liver) was 3000 U/mg protein and that of catalase (from bovine liver) was 3650 U/mg protein.

2.2. *Detection of chemiluminescence*

The rat heart under perfusion was enclosed in a light-tight perfusion box. The box was designed so that all manipulations could be carried out from the outside eliminating artifacts arising out of exposure of the photomultiplier to light. Chemiluminescence was measured by an EMI 9658 photomultiplier cooled to −20°C, at an applied potential of 0.95 kV. Standard high performance photon counting electronics consisting of a low-noise preamplifier, amplifier, discriminator and ratemeter were used. Dark counts were around 60 cps.

2.3. *Ischemia protocol*

The hearts were perfused for 10 min with the KH buffer. Then, the perfusion buffer was changed to KH buffer containing 0.2 μM Luminol (KHL buffer) for the rest of the experiment. Five of the hearts were subjected to 30 min of global ischemia by stopping the flow of the perfusion buffer. The ischemic period was followed by reperfusion for 1 h (Group I). In separate experiments, superoxide dismutase (SOD) (30 U/ml of perfusion buffer, 5 hearts) or catalase (CAT) (100 U/ml of perfusion buffer, 5 hearts) was administered for 10 min before and 30 min following ischemia along with the Luminol perfusion buffer (Group II and Group III, respectively).

The LEC intensity and the physiological parameters were monitored continuously through the course of the experiment.

3. RESULTS

Fig. 1 shows the chemiluminescence response of KH perfused and KHL perfused isolated rat heart to ischemia and reperfusion. The KH perfused heart shows very weak chemiluminescence that decreases on the onset of ischemia; the chemiluminescence recovers on reperfusion. However, the signal-to-noise ratio is too low for any detailed analysis. The KHL perfused heart shows a vastly improved signal-to-noise ratio and the time course of the changes in LEC can be clearly and quantitatively followed.

The LVDP, LVSP and CF values of the hearts did not change significantly on changing from KH to KHL buffer; following these parameters in control isolated perfused hearts over several hours did not show any differences between the KH perfused and KHL perfused hearts. No significant differences were seen between the 3 groups in terms of LVSP, LVDP or CF behavior in response to the 30 min ischemia–reperfusion. This suggests that the inclusion of Luminol in the perfusion buffer did not significantly affect the monitored physiological variables of the hearts.

The reproducibility of the changes in LEC, expressed as percent changes from their pre-ischemic value, was good with deviations of not more than 10% within any group. Fig. 2 shows typical LEC traces of hearts during ischemia–reperfusion when perfused with KHL buffer, KHL-SOD buffer and KHL-CAT buffer. The heart

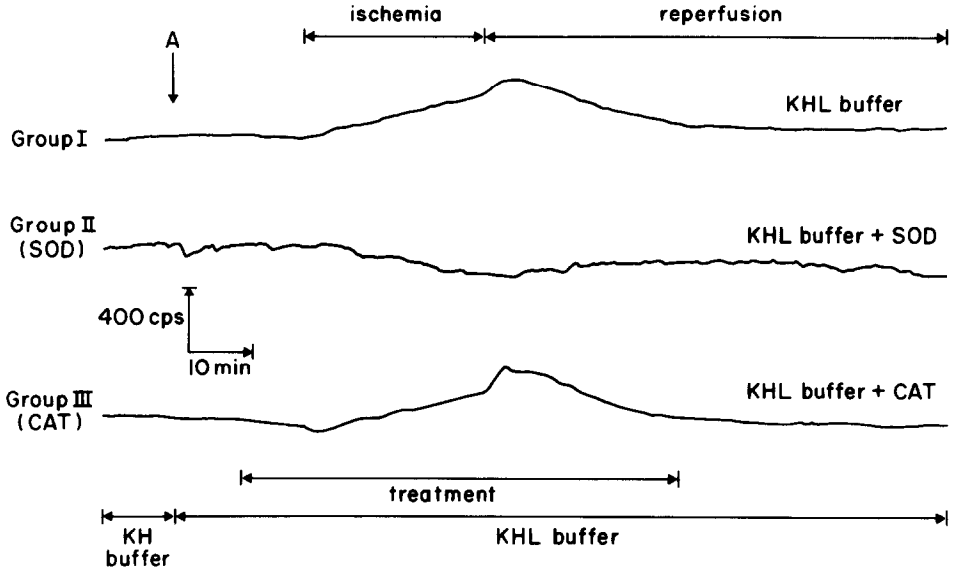

Fig. 1. Comparison of isolated perfused rat heart chemiluminescence changes in response to ischemia–reperfusion. Trace A; heart perfused with KH buffer (no Luminol). Trace B: heart perfused with KHL buffer (with Luminol).

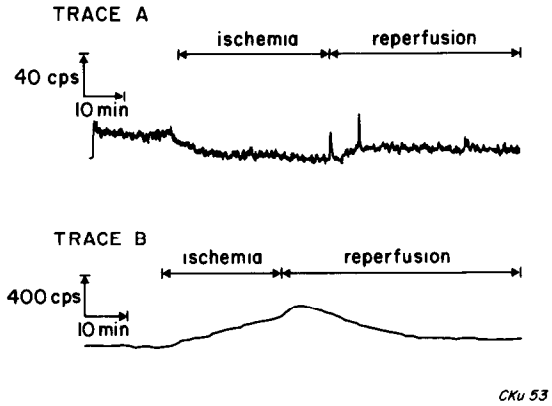

Fig. 2. Typical Luminol-enhanced chemiluminescence responses of the isolated rat heart perfused with KHL buffer. Group I, heart with no additional chemical treatment; Group II, heart with SOD treatment; Group III, heart with CAT treatment. For all the hearts, KH buffer was replaced by KHL buffer 10 min after the beginning of the measurement, at point A; 'treatment' shows the period through which SOD or CAT was administered.

perfused in KHL buffer shows an increase in LEC beginning at about 15 min of ischemia and reaches a value about 70% higher than the pre-ischemic level; reperfusion leads to a further increase in LEC to about 120% above the pre-ischemic level. In the heart perfused with KHL-SOD buffer, the increases in LEC are entirely abolished; further, the LEC levels during ischemia are lower than the pre-ischemic levels. The hearts perfused with KHL-CAT buffer showed behavior similar to the hearts perfused with KHL buffer.

4. DISCUSSION

The ischemia–reperfusion protocol used in this series of experiments was mild enough that there were no permanent changes in LVDP, LVSP or CF on reperfusion. Hence, if oxygen radicals are the agents causing ischemia–reperfusion damage, they were produced only in lower concentrations than necessary to cause serious tissue damage. The experimental system is sufficiently sensitive that we are able to follow the production of oxygen radicals through LEC measurements continuously and with good signal-to-noise ratios and statistics. It may be noted that the LEC intensities presented are an instantaneous measure of the oxygen radical concentration. Integrated over time, the LEC values provide a quantitative measure of total oxygen radical production.

SOD is effective in abolishing the large increases in LEC during ischemia and reperfusion while CAT is not. Thus, this experimental system is capable of evaluating the effect of chemical intervention on oxygen radical production in tissue. This model will thus be useful in screening chemicals that have been suggested to be of value in limiting ischemia–reperfusion induced damage [1,4]. A similar protocol can also be applied to other tissues such as perfused liver (Okuda, M., Ikai, I., Chance, B. and Kumar, C., manuscript in preparation).

Why is SOD so effective in abolishing LEC increases in the perfused heart during ischemia–reperfusion but not CAT? The first possibility is that the oxygen radical that is produced in significant amounts is superoxide; hydrogen peroxide is produced only at rates well below the maximal rates at which the tissue is capable of converting peroxide to oxygen or oxygen radicals so that added CAT has little effect on LEC intensities. The second possibility, that Luminol is sensitive only to superoxide and not to peroxide is excluded from the studies of mechanism of Luminol chemiluminescence, though some selectivity towards superoxide is known [19,23].

Lastly, it may be noted that significant production of oxygen radicals occurred in our studies not only during reperfusion but also during ischemia. Despite nominal global ischemia, enough oxygen enters the tissue, possibly assisted by myoglobin, through diffusion from the air that the condition is similar to low-flow ischemia and, it seems, conducive to superoxide production. Tissue oxygen pO_2 of the order of 1 mM Hg has been shown to be sufficient for the production of radicals in the ischemic myocardium [24].

Acknowledgements: We are indebted to Dr H.H. Seliger for many valuable suggestions and to Dr D. Smith and Dr M. Osbakken for reading the manuscript. We also thank the referee of this paper for relevant suggestions. This work was supported in part by NIH Grants HL18708 and AA07186.

REFERENCES

[1] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, 2nd edn, Clarendon Press, Oxford.
[2] McCord, J.M. (1985) N. Engl. J. Med. 312, 159–163.
[3] McCord, J.M. (1987) Fed. Proc. 46, 2402–2406.
[4] Opie, L.H. (1989) Circulation 80, 1049–1062.
[5] Myers, C.L., Weiss, S.J., Kirsh, M.M. and Schlafer, M. (1985) J. Mol. Cell. Cardiol. 17, 675–684.
[6] Simpson, P.J., Mickelson, J.K. and Lucchesi, B.R. (1987) Fed. Proc. 46, 2413–2421.
[7] Zweier, J.L., Kuppusamy, P. and Lutty, G.A. (1988) Proc. Natl. Acad. Sci. USA 85, 4046–4050.
[8] Zweier, J.L., Kuppusamy, P., Williams, R., Rayburn, B.K., Smith, D., Weisfeldt, M.L. and Flaherty, J.T. (1989) J. Biol. Chem. 264, 18890–18895.
[9] Baker, J.E., Felix, C.C., Olinger, G.N. and Kalyanaraman, B. (1988) Proc. Natl. Acad. Sci. USA 85, 2786–2789.
[10] Baker, J.E. and Kalyanaraman, B. (1989) FEBS Lett. 244, 311–314.
[11] Garlick, P.B., Davies, M.J., Hearse, D.J. and Slater, T.F. (1987) Circ. Res. 61, 757–780.
[12] Culcasi, M., Pietri, S. and Cozzone, P.J. (1989) Biochem. Biophys. Res. Commun. 164, 1274–1280.
[13] Bolli, R., Jeroudi, M.O., Patel, B.S., Dubose, C.M., Lai, E.K., Roberts, R. and McCay, P.B. (1989) Proc. Natl. Acad. Sci. USA 86, 4695–4699.
[14] Bolli, R. and McCay, P.B. (1990) Free Radical Res. Commun. 9, 169–180.

[15] Cadenas, E. (1989) J. Biolumin. Chemilumin. 4, 208–218.
[16] Boveris, A., Cadenas, E., Reiter, R., Filipowski, M., Nakase, Y. and Chance, B. (1980) Proc. Natl. Acad. Sci. USA 77, 347–381.
[17] Cadenas, E. and Sies, H. (1984) Methods Enzymol. 105, 221–230.
[18] Allen, R.C. and Loose, L.D. (1976) Biochem. Biophys. Res. Commun. 69, 245–253.
[19] Roswell, D.F. and White, E.H. (1978) Methods Enzymol. 57, 409–423.
[20] Dahlgren, C. (1989) Free Radic. Biol. Med. 6, 399–403.
[21] Johansson, A. and Dahlgren, C. (1989) J. Leukoc. Biol., 45, 444–451.
[22] Dahlgren, C., Follin, P., Johansson, A., Lock, R. and Orselius, K. (1989) J. Biolumin. Chemilumin. 4, 263–266.
[23] Hodgson, E.K. and Fridovich, I. (1973) Photochem. Photobiol. 18, 451–455.
[24] Rao, P.S., Cohen, M.V. and Mueller, H.S. (1983) J. Mol. Cell. Cardiol. 15, 713–716.